REMARKS

Claims 1 and 7 have been amended for clarification. It is realized that the designations of first and second nucleotide sequences were sometimes confused, as was certain other terminology. The claims now read appropriately, in applicants view. Further, to expedite prosecution, "consisting essentially of" has been deleted from claim 1 and the method is characterized as "consisting of" the steps shown.

It is believed that claim 1, as amended is clearly distinct from the disclosure of Katz. Attention is called to the requirement that the first nucleotide sequence and the second nucleotide sequence must both be functional PKS. There is no disclosure in Katz of using restriction enzymes and ligation to replace an acyl transferase domain in a sequence encoding a first functional PKS to produce a sequence encoding functional PKS.

Support for the requirement that both the unmodified and modified PKS undergoing a change in an AT domain must be functional is found, for example, on page 21 of the specification, lines 21-30. Both starting and resulting plasmids encode a functional PKS. This is illustrated by the triketide product produced by the modified form. The unmodified form contained in pCK12, also contains a nucleotide sequence encoding a functional PKS (PDEBS1).

A copy of the cited article (Kao, *et al.*, *JACS* (1995) 117:9105-9106) which describes pCK12 as encoding DEBS1 fused to TE on page 9105, first full paragraph in the right-hand column is enclosed to verify this.

Thus, no new matter is added and entry of the amendment is respectfully requested.

The Rejection Under 35 U.S.C. § 112, First Paragraph

Claims 1-5 and 31 were rejected under this paragraph as assertedly not in compliance with the written description requirement. The Office asserts that the claims have been broadened by adding the term "essentially" to "consisting." However, the word "consisting" never existed in the claims, and thus addition of the word "essentially" does not broaden the claim. That having been said, as this further limitation is believed not to be needed, the offensive term has been deleted from the claim.

Accordingly, it is believed this basis for rejection may be withdrawn.

The Rejection Under 35 U.S.C. § 102

Claims 1-5 and 31 were rejected as assertedly anticipated by Katz (WO93/13663) as well as Katz (U.S. 5,824,513). Applicants and the Office agree that these disclosures are essentially equivalent.

Applicants find nothing in Katz wherein an AT domain is excised from a first functional PKS and replaced by another AT domain using a different extender unit to obtain directly a modified form of the first PKS which is also functional. Restriction enzymes and ligations are employed in Katz, but never with respect to a functional PKS. For example, it appears that the restriction enzyme/ligation technique can be used to modify cloned portions of the DEBS-encoding nucleic acid, but in order to obtain sequences encoding functional PKS, these are reinserted by recombination into nucleotide sequences encoding functional PKS. This is made clear in column 5, beginning at line 21, where specific mutations in the eryA region of *Sac. erythraea* are provided by introduction of a specified change in a cloned DNA fragment, (non-functional) which is followed by exchange of the wildtype allele with a mutated one to obtain functionality. Only the first step

requires "standard recombinant DNA manipulations" employing *E. coli* as the host. In step 2, recombination techniques are used.

If the Office is able to find any disclosure in Katz of excising a region encoding an acyl transferase using restriction enzymes and straight ligation of an excised different AT encoding region where both the modified and unmodified nucleotide sequences encode functional PKS, applicants believe they are entitled to be directed to that portion of Katz. Applicants themselves are unable to find any such disclosure. Thus, it is believed this basis for rejection may be withdrawn.

The Obviousness Rejection

All pending claims were rejected as assertedly obvious over the two Katz documents described above in combination with Kao, *et al., Science* (1994) 265:509-512. The distinctions between Katz and claims 1-5 and 31 are set forth above. Kao is irrelevant to these claims.

As to claims 7 and 10-13, there is no suggestion in Kao that the techniques described therein could be used to exchange only portions of individual modules of modular PKS-encoding nucleotide sequences. Kao simply describes a technique for inserting the complete modules of DEBS-encoding nucleotide sequences into an expression vector. There is no suggestion to alter the nucleotide sequence encoding a module by use of the method of Kao. Inserting the complete DEBS sequence, or indeed a complete module, presents an entirely different problem from that of the present invention. In order to effect the method of the present invention, it would be necessary to modify the donor PKS-encoding nucleotide sequence so that the region to be donated is flanked by sequences homologous to the target location in the recipient. Kao, in combination with Katz, does not provide instructions to do this, and thus the combination falls short of suggesting the invention of claims 7 and 10-13. Accordingly, this basis for rejection, too, may be withdrawn.

Conclusion

The claims have been amended so as no longer to be subject to the rejection under § 112. The Office has not pointed to any disclosure in Katz whereby a functional PKS has been directly modified using restriction enzymes and ligation techniques to obtain a functional modified form that uses a different extender unit. Accordingly, claims 1-5 and 31 are not anticipated by Katz. Further, the combination of Kao with Katz does not result in the invention of claims 7 and 10-13 since neither Kao nor Katz provide instructions to provide homologous flanking sequences for the exchanged portions where they do not already exist in the nucleotide sequences to be exchanged. Thus, applicants believe that all pending claims are in a position for allowance and passage of these claims to issue is respectfully requested.

Should minor issues remain that could be resolved by phone, a telephone call to the undersigned is respectfully requested.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorize the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket No. 300622000508.

Respectfully submitted,

Dated: February 26, 2008

By: / Kate H. Murashige /

Kate H. Murashige
Registration No.: 29,959
MORRISON & FOERSTER LLP
12531 High Bluff Drive, Suite 100
San Diego, California 92130-2040
Telephone: (858) 720-5112
Facsimile: (858) 720-5125

Manipulation of Macrolide Ring Size by Directed Mutagenesis of a Modular Polyketide Synthase

Camilla M. Kao,† Guanglin Luo,‡ Leonard Katz,§ David E. Cane,‡ and C. Khosla*,†

Department of Chemical Engineering Stanford University, Stanford, California 94305-5025 Department of Chemistry, Brown University Box H, Providence, Rhode Island 02912 Antiinfective Discovery Research, Abbott Laboratories Abbott Park, Illinois 60064

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Polyketides are a huge family of natural products well-known for their antibiotic, antifungal, antiparasitic, immunosuppressive, and antitumor activities.[1] Polyketides are synthesized in a manner analogous to fatty acid biosynthesis,[2] in which carbon chains are built by successive decarboxylative condensations between coenzyme A (CoA) thioesters of organic acids. Structural diversity in this family of natural products arises during biosynthesis from varying choices of organic acid monomers, extents of β-carbon processing after each condensation reaction (to carbonyl, hydroxyl, enoyl, or methylene groups), stereochemistries of chiral carbon centers, and regiochemistries of cyclizations that occur after chain synthesis.[1,3]

Modular polyketide synthases (PKSs) are large multifunctional proteins (MW > 150 000)[4-6] that participate in the biosynthesis of macrolide antibiotics such as erythromycin and methymycin[7] (Figure 1). Genetic analysis of the 6-deoxyerythronolide B synthase (DEBS)[4,5] from *Saccharopolyspora erythraea*, which gives rise to the erythromycin aglycon, 6-deoxyerythronolide B (6dEB) (**1**), has suggested that all the active sites required for one cycle of condensation and β-ketoreduction are distinct and clustered as "modules"[4] (Figure 2). Early support for this model came from directed mutagenesis experiments on DEBS reductive sites[4,8,9] as well as the incorporation of chemically synthesized intermediates into 6dEB by *S. erythraea*.[10]

In an attempt to understand the relationship between structure and function in modular PKSs and to progress toward the rational and combinatorial design of novel polyketides, we developed a host–vector expression system, based on *Streptomyces coelicolor* CH999/pCK7, to study DEBS[11] (Figure 2). We recently demonstrated the production of (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**2**) (1–3 mg/L), the expected triketide product of the first two modules, by CH999/pCK9, which expresses DEBS1 alone[12] (Figure 2). This result provided further biochemical evidence for the modular PKS model of Katz and co-workers[4] and the unessential role of the thioesterase for enzyme product release.[13]

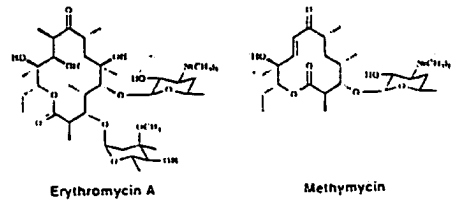

Figure 1. Macrolide antibiotics erythromycin A and methymycin.

Here, we analyze the role of the thioesterase (TE) domain from DEBS3 with two additional deletion mutants of DEBS. The first PKS, expressed by CH999/pCK12, contains DEBS1 fused to the TE. The fusion in this bimodular PKS occurs between the carboxy-terminal end of the acyl carrier domain of module 2 (ACP-2) and the carboxy-terminal end of the acyl carrier domain of module 6 (ACP-6)[14] (Figure 2). The second PKS, expressed by CH999/pCK15, includes the first four DEBS modules, a recombinant fifth module that is a hybrid between the wild-type modules 5 and 6, and the TE. The fusion in this pentamodular PKS exists 76 amino acids downstream of the β-ketoreductase of module 5 (KR-5) and five amino acids upstream of ACP-6[15] (Figure 2). Plasmids pCK12 and pCK15 were introduced into *S. coelicolor* CH999[16] and polyketide products purified from the transformed strains according to methods previously described.[11]

CH999/pCK12 produced **2** (20 mg/L) as determined by ^{1}H and ^{13}C NMR spectroscopy (Figure 2). This triketide product is identical to that produced by CH999/pCK9[12] but is produced in significantly greater quantities by CH999/pCK12 (~1 mg/L vs >20 mg/L). CH999/pCK12 also produced significant quantities of a novel analog of **2**, (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-hexanoic acid δ-lactone (**3**) (10 mg/L), that arises from the incorporation of an acetate start unit instead of propionate. This is reminiscent of the ability of CH999/pCK7, which expresses the wild-type DEBS1, DEBS2, and DEBS3 proteins, to produce 8,8a-deoxyoleandolide in addition to 6dEB.[11] The increased production of **2** as well as the facile isolation of **3** from CH999/pCK12 demonstrates the increased turnover rate of DEBS1 due to the TE. Thus the TE can effectively recognize an intermediate bound to a "foreign" module that is four acyl units shorter than its natural substrate, 6dEB. Recently, this increased triketide turnover rate was also independently observed with a similar DEBS1 mutant constructed in *S. erythraea*.[17] However, since the triketide products can probably cyclize spontaneously into **2** and **3** under typical fermentation conditions (pH 7), it is not possible to discriminate between enzyme-catalyzed cyclization and enzyme-catalyzed hydrolysis followed by spontaneous lactonization. Thus the ability of the TE to recognize the C-5 hydroxyl of a triketide as an incoming nucleophile is unclear.

† Stanford University.
‡ Brown University.
§ Abbott Laboratories.

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(14) Plasmid pCK12 contains *ery*A DNA originating from pS1 (Tuan, J. S.; *et al. Gene* 1990, *90*, 21). pCK12 is identical to pCK7[11] except for a deletion between the carboxy-terminal ends of ACP-2 and ACP-6. The fusion occurs between residues L3455 of DEBS1 and Q2891 of DEBS3. An *Spe*I site engineered between these two residues results in the DNA sequence CTCACTAGTCAG at the fusion.
(15) Plasmid pCK15 contains *ery*A DNA originating from pS1 (Tuan, J. S.; *et al. Gene* 1990, *90*, 21). pCK15 is a derivative of pCK7 and was constructed using an in vivo recombination strategy described earlier.[11] pCK15 is identical to pCK7 with the exceptions of a KR-5 to ACP-6 deletion, which occurs between residues G1372 and A2802 of DEBS3, and the insertion of a blunted *Sal*I fragment containing a kanamycin resistance gene (Oka, A.; *et al. J. Mol. Biol.* 1981, *147*, 217) into the blunted *Hin*dIII site of pCK7. An arginine residue is present between G1372 and A2802 so that the DNA sequence at the fusion is GGCCGCGCC.
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Exhibit 1

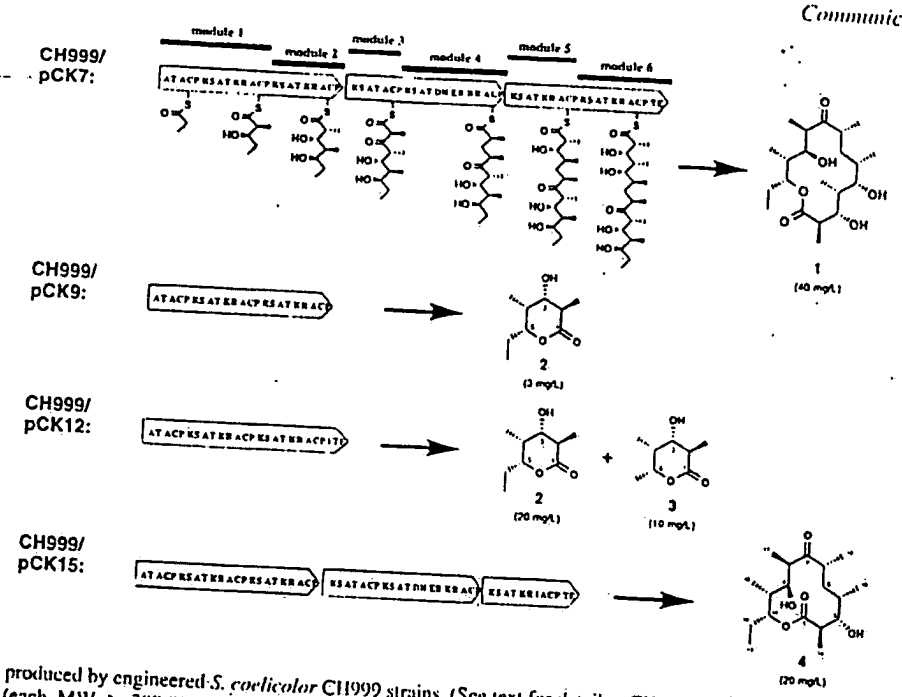

Figure 2. Polyketides produced by engineered *S. coelicolor* CH999 strains. (See text for details.) CH999/pCK7[11] expresses the polypeptides DEBS1, DEBS2, and DEBS3 (each MW > 300 000) that constitute DEBS.[4] KS: β-ketoacyl-acyl carrier protein synthase. AT: acyltransferase. DH: dehydratase. ER: enoyl reductase. KR: β-ketoreductase. ACP: acyl carrier domain. TE: thioesterase. 4 was characterized using ^{1}H and ^{13}C NMR spectroscopy, propionate-1-^{13}C and propionate-1,2,3-$^{13}C_3$ (sodium salts, Cambridge Isotopes) isotope labeling according to methods described previously,[11] homonuclear correlation spectroscopy (COSY), and heteronuclear correlation spectroscopy (HETCOR). 3: R_f = 0.32 (50% EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (t, 3H, J = 7.5 Hz, C_{13}-H_3), 1.00 (d, 3H, J = 6.6 Hz, C_{15}-H_3), 1.01 (d, 3H, J = 7.4 Hz, C_{18}-H_3), 1.12 (d, 3H, J = 6.9 Hz, C_{17}-H_3), 1.19 (d, 3H, J = 7.2 Hz, C_{16}-H_3), 1.26 (d, 3H, J = 6.7 Hz, C_{14}-H_3), 1.1–1.2 (1H, C_4-H), 1.40 (ddd, 1H, J = 3.5, 13.4, and 13.4 Hz, C_5-He), 1.48 (m, 1H, J = 7.0, 7.2, and 14.1 Hz, C_{12}-H), 1.63 (m, 1H, J = 7.0, 7.3, and 14.0 Hz, C_{12}-H), 1.79 (ddd, 1H, J = 2.1, 13.3, and 13.3 Hz, C_5-Ha), 1.91 (ddq, 1H, J = 2.7, 2.7, and 7.3 Hz, C_{10}-H), 2.55 (m, 1H, J = 3.6, 7.0, and 13.9 Hz, C_6-H), 2.64 (dq, 1H, J = 6.7 and 10.4 Hz, C_2-H), 3.12 (q, 1H, J = 6.8 Hz, C_8-H), 3.53 (d, 1H, J = 10.3 Hz, C_3-H), 3.53 (s, 1H, C_9-H), 4.97 (dt, 1H, J = 2.7 and 7.2 Hz, C_{11}-H); ^{13}C NMR of propionate-1,2,3-$^{13}C_3$-labeled 4 (100 MHz, $CDCl_3$) δ 8.92 (d, J = 35.4 Hz, C_{18}), 10.00 (d, J = 34.4 Hz, C_{13}), 12.28 (d, J = 33.2 Hz, C_{17}), 15.75 (d, J = 34.4 Hz, C_{14}), 17.38 (d, J = 35.4 Hz, C_{15}), 19.04 (d, J = 32.0 Hz, C_{16}), 24.40 (dd, J = 34.4 and 38.4 Hz, C_{12}), 33.15 (d, J = 32.8 Hz, C_5), 34.28 (dd, J = 34.8 and 38.3 Hz, C_4), 39.89 (dd, J = 33.2 and 37.3 Hz, C_8), 40.41 (dd, J = 36.0 and 40.5 Hz, C_{10}), 43.70 (dd, J = 34.3 and 56.4 Hz, C_2), 46.78 (dd, J = 32.6 and 32.8 Hz, C_6), 73.10 (d, J = 40.9 Hz, C_9), 78.46 (d, J = 38.1 Hz, C_3), 79.13 (d, J = 38.7 Hz, C_{11}), 175.31 (d, J = 54.5 Hz, C_1), 221.49 (d, J = 37.7 Hz, C_7); HR FAB MS, $[M + Na]^+$ 351.2147 calcd *m/e*, 351.2140 obsd *m/e*. Dihedral angles of aglycon protons of 4 modeled using Discover (CVFF force field, Biosym Technologies): C_2-H/C_3-H, 178°; C_3-H/C_4-H, −73°; C_4-H/C_5-Ha, −81°; C_4-H/C_5-He, 168°; C_5-Ha/C_6-H, 179°; C_5-He/C_6-H, −69°; C_8-H/C_9-H, 78°; C_9-H/C_{10}-H, 74°; C_{10}-H/C_{11}-H, −58°.

The second recombinant strain, CH999/pCK15, produced abundant quantities of (8*R*,9*S*)-8,9-dihydro-8-methyl-9-hydroxy-10-deoxymethynolide (4) (20 mg/L) (Figure 2). 4 is an analog of 10-deoxymethynolide,[18] the aglycon of the macrolide antibiotic methymycin (Figure 1). The production of 4 demonstrates that active site domains in modules 5 and 6 in DEBS can be joined without loss of activity. Moreover, the deletion of a single module from a wild-type bimodular polypeptide (DEBS3) provides further evidence for the structural and functional independence of individual modules as well as active sites in modular PKSs. Most remarkably, the formation of a 12-membered lactone ring via esterification of the terminal carboxyl with the C-11 hydroxyl suggests that the TE can catalyze lactonization of a polyketide chain one acyl unit shorter than 6dEB. Indeed, the formation of 4 may mimic the biosynthesis of the closely related 12-membered hexaketide macrolide, methymycin, which frequently co-occurs with the homologous 14-membered heptaketide macrolides, picromycin and/or narbomycin.[19] A modular PKS such as DEBS could thus possibly be used to generate a wide range of macrolactones with shorter as well as longer chain lengths, although the range of chain lengths that could undergo catalyzed lactonization remains unclear. Further experiments should shed more light onto the limits of TE specificity.

The construction of the pentamodular PKS has led to the biosynthesis of a previously uncharacterized 12-membered macrolactone that closely resembles, but is distinct from, the aglycon of a biologically active macrolide. The apparent structural and functional independence of active site domains and modules as well as relaxed lactonization specificity suggests the existence of many degrees of freedom for generating new modular PKSs. Libraries of new macrolides could be produced by altering the association of active site domains and modules, the subset of reductive sites within each module, the activity of the TE, and possibly even downstream modifications such as hydroxylation and glycosylation. Given the rich history of pharmacological activities among naturally occurring macrolides, such libraries could prove to be rich sources of new leads for drug discovery.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra and detailed analysis of spectral and modeling data of 4 (19 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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